

NOVEL ANTIBACTERIAL AGENTS AND METHODS OF IDENTIFYING
AND UTILIZING SAME

5 Inventors: Ehud GAZIT and Izhack CHERNY

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to methods of identifying antibacterial agents and more particularly, to novel antibacterial agents which are capable of preventing
10 or disrupting binding between antitoxin and toxin polypeptides of bacterial cells.

Presently, treatment of infections caused by pathogenic bacteria relies predominantly on the administration of antibiotics. Antibiotics currently being used against bacterial pathogens include β -lactams (e.g., penicillin and cephalosporin) and glycopeptides (e.g., vancomycin and teichoplanin), which act to inhibit the final step
15 in peptidoglycan synthesis, quinolones, which inhibit bacterial DNA replication, inhibitors of bacterial RNA polymerase such as rifampin, and aminoglycosides (e.g., kanamycin and gentamycin). Other well-known antibiotics include inhibitors of enzymes participating in production of tetrahydrofolate (e.g., sulfonamides).

Despite being successful in controlling or eliminating bacterial infections,
20 widespread use of antibiotics both in human medicine and as a feed supplement in poultry and livestock production has led to drug resistance in many pathogenic bacteria (McCormick J. B., Curr Opin Microbiol 1:125-129, 1998) and as such, the effectiveness of such antibiotics has greatly diminished in the last decade.

The rapid and widespread development of resistance in pathogenic bacteria
25 is illustrated by the fact that presently almost half of the clinical strains of *Haemophilus ducreyi*, the causative agent of chancroid, carry genes which confer resistance to amoxicillin, ampicillin and a series of other β -lactams (Prachayasittikul *et al.*, Southeast Asian J Trop Med Public Health 31:80-84, 2000). Likewise, the incidence of resistance towards tetracyclines among clinical strains of *Salmonella typhimurium* has increased from zero in 1948 to 98% by 1998 (Teuber M., Cell Mol
30 Life Sci 30:755-763, 1999).

The economic impact of managing infections caused by antibiotic-resistant bacteria is substantial, and current costs are estimated to be more than \$4 billion annually [Harrison and Lederberg (ed.), Antimicrobial resistance: issues and

options. National Academy Press, Washington, D.C. pp. 1-7, 1998]. Furthermore, as resistance spreads among bacteria, there is grave concern that antibiotics treatment will become increasingly less effective and, in some cases, completely ineffective.

This rapidly increasing appearance of bacterial resistance to antibiotics has driven researchers to search for new agents that possess activity against antibacterial drug-resistant strains. Although several approaches can be utilized to achieve this goal, the most generalized would be the discovery and clinical development of an agent that acts on a new target which has not yet experienced selective pressure in the clinical setting. Such a target should be essential to the growth and survival of bacteria and be sufficiently different from similar macromolecules present in the human host (Goldman and Gange, *Curr Med Chem* 7:801-820, 2000).

The Toxin-antitoxin complex of bacteria includes a pair of polypeptides that is encoded by bacterial plasmids and chromosomes. It is postulated that in bacteria these polypeptides function to induce programmed cell death or growth inhibition in response to starvation or other adverse conditions (Hayes, *Science* 301:1496-1499, 2003). The antitoxins neutralize the cognate toxins by forming tight complexes therewith. The antitoxins are unstable due to degradation by cellular proteases (e.g., Lon or Clp), whereas toxins are stable polypeptides. Toxin-antitoxin pair examples include the *pemI-pemK* genes of plasmid R100, the *phd-doc* genes of phage P1, and the *ccdA-ccdB* genes of plasmid F (Couturier *et al.*, *Trends Microbiol.* 6:269-275, 1998; Engelberg-Kulka and Glaser, *Annu. Rev. Microbiol* 53:43-70, 1999; Jensen and K. Gerdes, *Mol. Microbiol.* 17:205-210, 1995). Toxin-antitoxin pairs are thought to increase the stability of extrachromosomal elements by selectively killing plasmid-free cells, resulting in the proliferation of plasmid-harboring cells in the population (Holcík and Iyer, *Microbiology* 143:3403-3416, 1997; and Grady and Hayes, *Mol. Microbiol.* 47:1491-1432, 2003). Several toxin-antitoxin encoding gene analogues have been identified on the *E. coli* K-12 chromosome, such as *mazE-mazF* (also known as *chpAI-chpAK*), *sof-gef*, *kicA-kicB*, *relB-relE*, *chpBI-chpBK* and *yefM-yoeB* (Grady and Hayes, *Mol. Microbiol.* 47:1491-1432, 2003; Aizenman *et al.*, *Proc. Natl. Acad. Sci. USA* 93:6059-6063, 1996; Feng *et al.*, *Mol. Gen. Genet.* 243:136-147, 1994; Gotfredsen and Gerdes, *Mol. Microbiol.* 29:1065-1076, 1998;

Masuda *et al.*, J. Bacteriol. 175:6850–6856, 1993; and Poulsen *et al.*, Mol. Microbiol. 3:1463–1472, 1989).

Although the use of toxin encoding polynucleotides for inducing bacterial cell death has been recently suggested (Westwater *et al.*, Antimicrobial Agents and Chemotherapy 47: 1301-1307, 2003), the prior art does not teach or suggest prevention or disruption of toxin-antitoxin binding for the purpose of inducing death in bacterial cells.

While reducing the present invention to practice, the present inventors have identified the site of interaction between bacterial toxin and antitoxin polypeptides thus enabling for the first time to identify or design novel antibiotics which target this site of interaction and thus enable bacterial cell killing.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of identifying a molecule capable of inducing death of a bacterial cell. The method includes (i) exposing toxin and antitoxin polypeptides of a toxin-antitoxin pair produced by the bacterial cell to a plurality of molecules; and (ii) identifying a molecule of the plurality of molecules capable of preventing or disrupting binding between the antitoxin and the toxin polypeptides.

According to another aspect of the present invention there is provided a method of treating an infection of bacteria in a subject. The method includes preventing or disrupting binding between a toxin and an antitoxin polypeptides of a toxin-antitoxin pair produced in the bacteria.

According to yet another aspect of the present invention there is provided a pharmaceutical composition for treating an infection of bacteria which includes an effective amount of an agent capable of preventing or disturbing binding between a toxin and an antitoxin polypeptides of a toxin-antitoxin pair produced in the bacteria.

According to still another aspect of the present invention there is provided a method of identifying toxin and antitoxin polypeptides of a toxin-antitoxin pair. The method includes (i) identifying bacterial polynucleotide sequences at least partially homologous to polynucleotide sequences encoding known bacterial toxin and antitoxin polypeptides to thereby obtain a plurality of toxin and antitoxin encoding

sequences; and (ii) determining a chromosomal position of each of the plurality of sequences, wherein toxin and antitoxin encoding sequences which are chromosomally positioned at a distance from each other which is no greater than a predetermined value encode a toxin-antitoxin pair.

5 According to further features in preferred embodiments of the invention described below, exposing toxin and antitoxin polypeptides of a toxin-antitoxin pair produced by the bacterial cell to a plurality of molecules is effected by administering the plurality of molecules to bacteria expressing the toxin and antitoxin polypeptides.

According to still further features in the described preferred embodiments, the
10 antitoxin polypeptide is an unfolded polypeptide.

According to still further features in the described preferred embodiments, the antitoxin polypeptide includes an amino acid sequence selected from the group consisting of SEQ ID NOs. 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122 and 124.

15 According to still further features in the described preferred embodiments, the antitoxin polypeptide is encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NOs. 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64 and 66.

According to still further features in the described preferred embodiments, the
20 toxin polypeptide includes an amino acid sequence selected from the group consisting of SEQ ID NOs. 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123 and 125.

According to still further features in the described preferred embodiments the toxin polypeptide is encoded by a polynucleotide sequence selected from the group
25 consisting of SEQ ID NOs. 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65 and 67.

According to still further features in the described preferred embodiments, preventing or disrupting binding between the toxin and the antitoxin polypeptides is effected by providing to the subject an agent selected from the group consisting of (i)
30 a compound which specifically binds to the antitoxin or to the toxin; (ii) an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the antitoxin; (iii) a ribozyme which specifically cleaves transcripts encoding the

antitoxin; and (iv) a small interfering RNA (siRNA) molecule which specifically cleaves the antitoxin transcripts.

According to still further features in the described preferred embodiments the compound is selected from the group consisting of a peptide, a polynucleotide, a polysaccharide, a small organic compound and a non-biological compound.

According to still further features in the described preferred embodiments the compound which specifically binds to the antitoxin is an antibody or an antibody fragment.

According to still further features in the described preferred embodiments the bacteria are pathogenic bacteria.

According to still further features in the described preferred embodiments the subject is a mammal.

According to still further features in the described preferred embodiments the subject is a human.

According to still further features in the described preferred embodiments the peptide is derived from the toxin or from the antitoxin.

According to still further features in the described preferred embodiments the peptide includes an amino acid sequence selected from group consisting of SEQ ID NOs: 7-9.

According to still further features in the described preferred embodiments, the agent is a polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the antitoxin.

According to still further features in the described preferred embodiments, the agent is a ribozyme which specifically cleaves transcripts encoding the antitoxin

According to still further features in the described preferred embodiments, the agent is a small interfering RNA (siRNA) molecule which specifically cleaves the antitoxin transcripts

According to still further features in the described preferred embodiments, the predetermined value is ranging between 10 base pair to 150 base pair.

The present invention successfully addresses the shortcomings of the presently known configurations by providing methods of identifying novel antibacterial agents which are capable of preventing or disrupting binding between

antitoxin and toxin polypeptides of bacterial cells and pharmaceutical compositions comprising these agents for treating bacterial infections.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 illustrates *yoeB* and *yefM* pair homologue sequences identified in different bacterial genomes. These homologue sequences were separated by less than 100 base pairs in their respective bacterial genomes and thus were selected for further analysis. The black half-ovals represent *yefM* homologues and the gray half-ovals represent *yoeB* homologue sequences. The sharp gray arrowheads represent *doc* homologue sequences. Missing gi numbers indicate annotated open reading frames (ORFs).

FIG. 2A illustrates a multiple sequence alignment of YefM polypeptide homologues. The alignment list includes 30 sequences from 25 different bacteria (different homologues which exist in the same bacteria are presented in alphabetical order). Polypeptide sections having sequence identity of $\geq 80\%$, $\geq 60\%$ and $\geq 40\%$ are colored in dark, medium and light blue background, respectively. The identity percentage values were determined by using a BLOSUM62 matrix.

FIG. 2B illustrates a multiple sequence alignment of YoeB polypeptide homologues. Polypeptide sections having sequence identity of $\geq 80\%$, $\geq 60\%$ and $\geq 40\%$ are colored in dark, medium and light blue background, respectively. The identity percentage values were determined by using a BLOSUM62 matrix. The upper alignment list illustrates 26 amino acid sequences obtained from 22 different

bacteria, all showing substantial homology to the YoeB polypeptide. The lower alignment list illustrates three Doc homologue sequences.

FIGs 3A-G illustrate the function of the YoeB and YefM polypeptides as a toxin-antitoxin pair *in vivo*. Bacteria (*E. coli* strain TOP10) expressing YefM only (Figures 3A and 3D), expressing YoeB only (Figures 3B and 3E), or expressing YoeB and YefM combined (Figures 3C and 3F), were grown in LB-Amp at 37 °C. Transcription of the polypeptides was induced by supplementing 0.2% L-arabinose (full circles) to the stationary growth phase at time zero (Figures 3A-C), or at the logarithmic growth phase, when cultures reached an OD₆₀₀ of 0.45 (Figures 3D-F). As a negative control, parallel cultures were supplemented with 0.2% glucose (open circles). Figure 3G illustrates the effect of overexpressing YefM alone, YoeB alone, or YoeB and YefM combined, on bacterial colony formation. Bacterial suspension droplets were added, in serial ten-fold dilutions, onto solid media supplemented with different concentrations of L-arabinose (0%, 0.0005%, 0.005%, 0.02%, 0.05%, 0.1%, and 0.2%) and incubated at 37°C for 20 hours. Cultures missing L-arabinose were supplemented with 0.2% glucose. The resulting colony formation of bacteria expressing YoeB alone was inversely proportional to L-arabinose concentrations, while colony formation of bacteria expressing YoeB and YefM combined was unaffected by the L-arabinose concentration.

FIGs. 4A-D illustrate the native unfolded state of the YefM antitoxin polypeptide. Figure 4A shows spectra of far UV circular-dichroism (CD) of YefM in PBS (pH 7.3) at 25 °C (---), 37 °C (—), and 42 °C (— —), indicating a random coil structure. Figure 4B illustrates spectra of Fourier Transform Infrared of the YefM polypeptide having a minimum transmittance at wave-number of 1643 cm⁻¹, indicating a random coil structure. Figure 4C illustrates the thermal stability of YefM at 2 °C to 80 °C temperature range. The thermal stability of YefM was determined by monitoring CD ellipticity at 217 nm (triangles) and 222 nm (circles) as a function of temperature. Figure 4D shows an SDS-PAGE analysis illustrating solubility of the YefM polypeptide which survived a boiling treatment. The left lane illustrates SDS-PAGE of YefM-GST sample following cleavage. The right lane illustrates an SDS-PAGE of the supernatant of the same sample following boiling for 10 minutes and centrifugation.

FIG. 5A illustrates the relative occurrence of amino acids in YefM family of polypeptides, in relation to the general occurrence of amino acids in polypeptide compositions [based on NCBI proteins database (22)]. Error bars represent standard deviations and the asterisks indicate statistically significant differences at $P < 0.001$. This figure illustrates that the YefM family of polypeptides are uniquely enriched in the amino acids M and E and uniquely depleted in the amino acids W, C, P, F and G.

FIG. 5B illustrates the calculated net charge and hydrophobicity of polypeptide homologues of YefM (circles) and Phd (triangles). The solid line represents Uversky *et al.* model (3) separating presumptive unfolded polypeptides (upper left area) from folded polypeptides (bottom right area).

FIGS. 6A-C are peptide array analyses identifying a YefM derivative which contains the antitoxin binding determinant. Figure 6A illustrates an array of tridecamer peptides corresponding to consecutive overlapping sequences of 92 amino acids of the YefM polypeptide (two amino-acids shift between peptides). A YoeB-GST bound to the membrane is positively identified in YefM₁₁₋₂₃–YefM₁₅₋₂₇, YefM₃₃₋₄₅, YefM₇₅₋₈₇ and YefM₇₇₋₈₉ peptides. Figure 6B illustrates a similar array of peptides corresponding to consecutive overlapping sequences of YefM₈₋₃₁, YefM₂₉₋₄₈, and YefM₇₂₋₉₂ (single amino-acid shift between peptide), indicating that the best binding of YoeB-GST was to YefM₁₁₋₂₃. Figure 6C illustrates of YefM₁₁₋₂₃ analogs having single amino acid replacements. YefM₁₁₋₂₃ analogs having arginine in position 19 of the YefM₁₁₋₂₃ sequence, which was replaced with either alanine or glycine, were unable to bind YoeB-GST.

FIGS. 7A-B illustrate the instability of the YefM antitoxin *in vivo*. Expression of YefM in *E. coli* TOP10 was briefly induced then repressed. Following repression, samples taken sequentially at different time intervals were analyzed for YefM expression by western blot analysis (Figure 7A) and by densitometer (Figure 7B), indicating a half-life of about one hour for this polypeptide.

FIG. 8 is a surface plasmon resonance (SPR) analysis illustrating binding of GST-YoeB fusion polypeptide to the YefM₁₁₋₂₃ peptide which contains the YefM binding determinant. SPR sensorgrams show the change in binding response (in RU) upon injection of 12.5, 25 and 50 nM of GST-YoeB in 50 mM Tris-HCl (pH

7.2) running buffer over the YefM₁₁₋₂₃ peptide.

FIGs. 9 A-C illustrate chromatography analyses indicating a tight binding between YoeB-His and YefM. Figure 9A shows a co-elution of YefM and YoeB-His in a nickel affinity column chromatography. Figure 9B shows a co-elution of YefM and YoeB-His resulting from an anion-exchange column developed with NaCl gradient. Figure 9C shows a co-elution of YefM and YoeB-His resulting from a C₁₈ HPLC reverse-phase column developed with acetonitrile gradient.

FIGs. 10A-C illustrate the structure and stability of the YoeB toxin. Figure 10A shows spectra of far UV circular-dichroism (CD) of 2.5 μ M YoeB-His monitored at 4 °C, 37 °C and 4 °C after 37 °C, indicating a predominant α -helical structure. Figure 10B shows spectra of Near-UV CD of 10 μ M YoeB-His monitored at 4° C and 37° C, indicating that no structural changes of the polypeptide occurred within that temperature range. Figure 10C illustrates the thermal stability of YoeB-His at thermal melt and return range of 2 \rightleftharpoons 80 °C. The thermal stability of YoeB-His was determined by monitoring CD ellipticity at 222 nm as a function of temperature.

FIGs. 11A-C illustrate the thermal melting point of YoeB. Figure 11A illustrates the CD ellipticity of YoeB-His as a function of temperature at 2 \rightleftharpoons 37 °C. Figure 11B illustrates the CD ellipticity of YoeB-His as a function of temperature at 2 \rightleftharpoons 56 °C. Figure 11C illustrates the CD ellipticity of YoeB-His as a function of temperature at 2 \rightleftharpoons 60 °C, indicating that YoeB undergoes thermal denaturation at approximately 60 °C.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods of identifying agents capable of preventing or disrupting binding between antitoxin and toxin polypeptides in bacterial cells, and of pharmaceutical compositions which include such agents and their use for treating bacterial infections.

The principles and operation of the present invention may be better understood with reference to the drawings, examples and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of

construction and the arrangement of the components set forth in the following description or illustrated in the drawings and the examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

As is discussed hereinabove, toxin-antitoxin polypeptide pairs are often present in bacterial cells. Although their function in such cells is not clear, it has been conclusively shown that antitoxin polypeptides bind to their cognate toxins to thereby neutralizing their cytotoxic activity in bacterial cells.

Using bioinformatic tools, the present inventors uncovered that homologues of the YoeB-YefM toxin-antitoxin pair exist in a wide range of bacteria, including several pathogenic bacteria. Biochemical analysis has revealed that the YefM antitoxin is an unfolded polypeptide in its native state within bacterial cells. Further analysis using an isolated peptide derivative of YefM (YefM₁₁₋₂₃; set forth in SEQ ID NO: 7) identified the determinant sequence of YefM which is involved in YoeB toxin binding; surprisingly, an alteration of just one amino acid of the YefM₁₁₋₂₃ is sufficient to completely abolish this binding (see in Examples 1-6 hereinbelow). It was thus concluded that binding between the YoeB toxin and the YefM antitoxin polypeptides relies upon a highly specific recognition which can be readily prevented or disrupted.

Thus, according to one aspect of the present invention, there is provided a method of treating a bacterial infection in a subject, such as a mammal, preferably, a human.

The method is effected by preventing or disrupting binding between toxin and antitoxin polypeptides of toxin-antitoxin pairs produced in the bacteria responsible for infection.

The bacteria can be any bacteria which produces a toxin-antitoxin pair. Preferably, the bacteria are *Enterococcus faecium*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Salmonella typhimarium*, *Streptococcus pneumoniae*, *Yersinia enterocolitica* and *E. coli*.

The toxin-antitoxin pair can be any pair of polypeptides encoded by a plasmid or chromosome of the bacteria responsible for the infection. Specific

examples of toxin-antitoxin pairs are provided in Table 1 and in the Examples section hereinbelow.

The phrase “preventing or disrupting” used herein refers to precluding binding between toxin and antitoxin polypeptides, or to disassociating a complex formed therefrom. As further described hereinbelow, such prevention or disruption can be effected by reducing expression of the antitoxin, or by reducing the antitoxin-toxin binding capacity.

Preventing or disrupting binding of a toxin-antitoxin pair can be effected using any one of several approaches.

In one approach, disrupting binding of a toxin-antitoxin pair is effected by providing to the subject an agent which specifically binds to the antitoxin, or, preferably, to the antitoxin binding determinant.

The binding determinant sequence of the antitoxin can be identified using techniques well known in the art including, but not limited to, peptide array analysis and surface plasmon-resonance analysis. Example 6 illustrates the isolation of a peptide derivative of the YefM antitoxin which includes the YefM antitoxin binding determinant (set forth in SEQ ID NO: 7). It will be appreciated that such characterization of the antitoxin binding determinant enables identification or design of a compound capable of preventing or disrupting the binding between toxin and antitoxin polypeptides. For example, using this determinant sequence, one of ordinary skill in the art can screen compound libraries for compounds which are capable of specifically binding this determinant and thus capable of preventing toxin-antitoxin binding.

An agent which specifically binds to the antitoxin binding determinant can be, for example, a peptide, a polynucleotide, a carbohydrate, a small organic molecule, or a non-biological compound.

The phrase “non-biological compound” used herein refers to an organic or an inorganic compound which is not naturally present in living organisms.

A peptide which specifically binds to the antitoxin binding determinant itself can be a toxin derivative which includes the toxin binding determinant sequence (the portion of the toxin molecule which participates the antitoxin-toxin binding). In cases where the antitoxin binding determinant is isolated, the toxin binding

determinant sequence can be readily identified using, for example, the peptide array analysis procedure described in Example 6 (by using toxin derivative peptides to form the array and a labeled antitoxin derivative as a probe).

Alternatively, a peptide, a polynucleotide, a carbohydrate, a small organic molecule, or a non-biological compound which specifically binds to the antitoxin binding determinant can be identified by using standard rational drug design methods or high throughput screening of combinatorial libraries, as described hereinbelow.

Disrupting binding between members of a toxin-antitoxin pair may also be effected by providing to the subject an agent (e.g., peptide) which specifically binds the toxin in a manner which interrupts toxin-antitoxin binding while at the same time does not substantially affect toxin activity. Toxin sequence regions (toxin binding determinants) which can be targeted by such an agent and agents which specifically bind thereto can be identified using methodology similar to that described above with respect to identifying antitoxin binding determinant sequences and agents which specifically bind thereto.

The agent identified capable of binding to the antitoxin or toxin binding determinant of infectious bacteria can be administered to the subject in need orally, intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally. A peptide or polynucleotide agent is preferably administered encapsulated in a suitable carrier, such as a liposome. Suitable encapsulated carriers may be prepared using methods such as described, for example, in U.S. Pat. No. 6,610,478, 6,309,669, 5,013,556 and 4,925,673 and by Clarenc *et al.* Anti-Cancer Drug Design, 8:81-94, 1993; Felgner, Advanced Drug Delivery Reviews, 5:163-187, 1990; and Wang *et al.*, Biochem. 28:9508-9514, 1989.

Delivery of a polynucleotide agent to target infecting bacteria may also be effected by utilizing a recombinant nonlytic phage, such as M13 (Westwater *et al.*, Antimicrobial Agents and Chemotherapy 47: 1301-1307, 2003).

Delivery of carbohydrates, small organic molecules, or non-biological compounds to target infecting bacteria in the subject may be effected using methods well known in the art such as described, for example, by Johnson *et al.*, eds. (Drug Delivery Systems, Chichester, England: Ellis Horwood Ltd., 1987). Additional

methods of formulating and administering pharmaceutical compositions are described hereinbelow.

Another agent which can be used to specifically inhibit toxin-antitoxin binding is an antibody or an antibody fragment.

5 Preferably, the antibody or antibody fragment specifically binds at least one epitope of the antitoxin binding determinant. As used herein, the term "epitope" refers to any antigenic determinant on an antigen to which the paratope of an antibody binds.

10 Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

 The phrase "antibody or an antibody fragment" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, 15 F(ab')₂, and Fv that are capable of binding to macrophages. These functional antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can 20 be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) F(ab')₂, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide 25 bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain 30 molecule.

 Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (see for example, Harlow and Lane,

Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988).

Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, in U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. (Biochem. J. 73: 119-126, 1959). Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar *et al.* (Proc. Natl Acad. Sci. USA 69:2659-2662, 1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde.

Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (scFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing scFv are described, for example, by Whitlow and Filpula (Methods 2: 97-105, 1991), Bird *et al.* (Science

242:423-426, 1988), Pack *et al.* (Bio/Technology 11:1271-1277, 1993) and in U.S. Pat. No. 4,946,778.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry (Methods, 2: 106-110, 1991).

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones *et al.*, Nature, 321:522-525, 1986; Riechmann *et al.*, Nature, 332:323-329, 1988; and Presta, Curr. Op. Struct. Biol., 2:593-596, 1992).

Methods for humanizing non-human antibodies or antibody fragments are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed

following the method of Jones *et al.* (Nature, 321:522-525, 1986), Riechmann *et al.* (Nature 332:323-327, 1988), and Verhoeyen *et al.* (Science, 239:1534-1536, 1988), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies or antibody fragments can also be produced using various techniques known in the art, including phage display libraries (Hoogenboom and Winter, J. Mol. Biol., 227:381, 1991; Marks *et al.*, J. Mol. Biol., 222:581, 1991). The techniques of Cole *et al.* and Boerner *et al.* are also available for the preparation of human monoclonal antibodies (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner *et al.*, J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al.*, Bio/Technology 10: 779-783 (1992); Lonberg *et al.*, Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild *et al.*, Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

The antibody or antibody fragment of the present invention can be delivered to the target infecting bacteria in the subject using methods known in the art. Preferably, the antibody or antibody fragment is administered contained in a microparticle or microencapsulated carrier such as described, for example, in U.S. Pat. Nos. 6,610,478, 6,309,669, 5,013,556 and 4,925,673 and by Clarenc *et al.* Anti-

Cancer Drug Design, 8:81-94, 1993; Felgner, Advanced Drug Delivery Reviews, 5:163-187, 1990; and Wang *et al.*, Biochem. 28:9508-9514, 1989.

Preventing or disrupting binding of a toxin-antitoxin pair may also be effected by providing to the subject an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the antitoxin.

Design of antisense molecules which can be used to efficiently hybridize with an mRNA transcript encoding the antitoxin must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which specifically binds the designated mRNA within cells in a way which inhibits translation thereof.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types [see, for example, Luft J Mol Med 76: 75-6 (1998); Kronenwett *et al.* Blood 91: 852-62 (1998); Rajur *et al.* Bioconjug Chem 8: 935-40 (1997); Lavigne *et al.* Biochem Biophys Res Commun 237: 566-71 (1997) and Aoki *et al.* (1997) Biochem Biophys Res Commun 231: 540-5 (1997)].

In addition, algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton *et al.* Biotechnol Bioeng 65: 1-9 (1999)].

Such algorithms have been successfully used to implement an antisense approach in cells. For example, the algorithm developed by Walton *et al.* enabled scientists to successfully design antisense oligonucleotides for rabbit beta-globin (RBG) and mouse tumor necrosis factor-alpha (TNF alpha) transcripts. The same research group has more recently reported that the antisense activity of rationally selected oligonucleotides against three model target mRNAs (human lactate dehydrogenase A and B and rat gp130) in cell culture as evaluated by a kinetic PCR technique proved effective in almost all cases, including tests against three different targets in two cell types with phosphodiester and phosphorothioate oligonucleotide chemistries.

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an *in vitro* system were also published (Matveeva *et al.*, Nature Biotechnology 16: 1374 - 1375 (1998)).

Several clinical trials have demonstrated safety, feasibility and activity of antisense oligonucleotides. For example, antisense oligonucleotides suitable for the treatment of cancer have been successfully used [Holmund *et al.*, Curr Opin Mol Ther 1:372-85 (1999)], while treatment of hematological malignancies via antisense oligonucleotides targeting c-myc gene, p53 and Bcl-2 had entered clinical trials and had been shown to be tolerated by patients [Gerwitz Curr Opin Mol Ther 1:297-306 (1999)].

An example of using antisense molecules to treat bacterial infection is described in U.S. Pat. No. 6,677,153. Accordingly, oligomers antisense to bacterial 16S or 23S rRNA are capable of selectively modulating the biological activity thereof and thus can be used as antibacterial agents.

Thus, the current consensus is that recent developments in the field of antisense technology which, as described above, have led to the generation of highly accurate antisense design algorithms and a wide variety of oligonucleotide delivery systems, enable an ordinarily skilled artisan to design and implement antisense approaches suitable for downregulating expression of known sequences without having to resort to undue trial and error experimentation. A suitable antisense sequence according to the teaching of the present invention can be, for example, the antisense to YefM antitoxin set forth in SEQ ID NO: 126.

Preventing or disrupting binding of a toxin-antitoxin pair may also be effected by providing a small interfering RNA (siRNA) molecule which specifically cleaves the antitoxin transcripts.

RNA interference is a two step process. The first step, which is termed as the initiation step, input dsRNA is digested into 21-23 nucleotide (nt) small interfering RNAs (siRNA), probably by the action of Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, which processes (cleaves) dsRNA (introduced directly or via a transgene or a virus) in an ATP-dependent manner. Successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNA), each with 2-

nucleotide 3' overhangs [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002); and Bernstein Nature 409:363-366 (2001)].

In the effector step, the siRNA duplexes bind to a nuclease complex to form the RNA-induced silencing complex (RISC). An ATP-dependent unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA into 12 nucleotide fragments from the 3' terminus of the siRNA [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002); Hammond et al. (2001) Nat. Rev. Gen. 2:110-119 (2001); and Sharp Genes. Dev. 15:485-90 (2001)]. Although the mechanism of cleavage is still to be elucidated, research indicates that each RISC contains a single siRNA and an RNase [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002)].

Because of the remarkable potency of RNAi, an amplification step within the RNAi pathway has been suggested. Amplification could occur by copying of the input dsRNAs which would generate more siRNAs, or by replication of the siRNAs formed. Alternatively or additionally, amplification could be effected by multiple turnover events of the RISC [Hammond et al. Nat. Rev. Gen. 2:110-119 (2001), Sharp Genes. Dev. 15:485-90 (2001); Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002)]. For more information on RNAi see the following reviews Tuschl ChemBiochem. 2:239-245 (2001); Cullen Nat. Immunol. 3:597-599 (2002); and Brantl Biochem. Biophys. Act. 1575:15-25 (2002).

Synthesis of RNAi molecules suitable for use with the present invention can be effected as follows. First, the target antitoxin mRNA sequence is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR

mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level (www.ambion.com/techlib/tn/91/912.html).

Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat etc.) using any sequence alignment software, such as the BLAST software available from the NCBI server (www.ncbi.nlm.nih.gov/BLAST/). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene. Suitable siRNA molecules according to the teaching of the preset invention include, for example, the siRNA to the YefM antitoxin set forth in SEQ ID NOs: 127-128.

Preventing or disrupting binding of a toxin-antitoxin pair may also be effected by providing a DNzyme molecule capable of specifically cleaving an mRNA transcript or DNA sequence of the antitoxin. DNzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl. Acad. Sci. USA 1997;94:4262) A general model (the "10-23" model) for the DNzyme has been proposed. "10-23" DNzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl. Acad. Sci. USA 1999; for rev of DNzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNzymes recognizing single and double-stranded target cleavage sites have been

disclosed in U.S. Pat. No. 6,326,174 to Joyce *et al.* DNazymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis in vivo (Itoh *et al.*, 20002, Abstract 409, Ann Meeting Am Soc Gen Ther www.asgt.org). In another application, DNazymes complementary to bcr-abl oncogenes were successful in inhibiting the oncogenes expression in leukemia cells, and lessening relapse rates in autologous bone marrow transplant in cases of CML and ALL.

Preventing or disrupting binding of a toxin-antitoxin pair may also be effected by providing a ribozyme which specifically cleaves transcripts encoding the antitoxin

Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding polypeptides of interest [Welch *et al.*, Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch *et al.*, Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms has demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

Essentially, binding of any toxin-antitoxin pair of any bacterium can be prevented or disrupted using the above described agents and methodology. Table 1

below provides examples of several toxin-antitoxin pairs which can be targeted by the present invention.

Table 1
Target bacterial toxin-antitoxin pairs

Bacterial species or strain *	Antitoxin		Toxin	
	Amino-acid SEQ ID NO	Polynucleotide SEQ ID NO	Amino-acid SEQ ID NO	Polynucleotide SEQ ID NO
<i>Actinobacillus actinomycetemcomitans</i>	68	10	69	11
<i>Agrobacterium tumefaciens</i>	70	12	71	13
<i>Burkholderia cepacia</i>	72	14	73	15
<i>Coxiella burnetii</i>	74	16	75	17
<i>Escherichia coli</i> (YefM/YoeB)	76	18	77	19
<i>Enterococcus faecium</i> pRUM plasmid	78	20	79	21
<i>Francisella tularensis</i> pFNL10 plasmid (Phd)	80	22	81	23
<i>Klebsiella pneumoniae</i>	82	24	83	25
<i>Mycobacterium bovis</i>	84	26	85	27
<i>Mycobacterium tuberculosis</i>	86	28	87	29
<i>Neisseria europea A</i>	88	30	89	31
<i>Neisseria europea B</i>	90	32	91	33
<i>Neisseria europea C</i>	92	34	93	35
<i>Nostoc sp.</i> PCC 7120	94	36	95	37
<i>Pseudomonas fluorescence</i>	96	38	97	39
<i>Pseudomonas putida</i>	98	40	99	41
<i>Pseudomonas syringae</i>	100	42	101	43
<i>Rickettsia conorii</i>	102	44	103	45
<i>Salmonella typhimarium</i>	104	46	105	47
<i>Streptococcus aureus</i>	106	48	107	49
<i>Streptococcus pneumoniae</i>	108	50	109	51
<i>Streptomyces coelicolor</i>	110	52	111	53
<i>Streptomyces viridochromogenes</i>	112	54	113	55
<i>Synechocystis sp.</i> PCC 7942	114	56	115	57
<i>Synechocystis sp.</i> PCC 6803 A	116	58	117	59
<i>Synechocystis sp.</i> PCC 6803 B	118	60	119	61
<i>Tiobacillus ferrooxidans</i>	120	62	121	63
<i>Yersinia enterocolitica A</i>	122	64	123	65
<i>Yersinia enterocolitica B</i>	124	66	125	67

5 * species which appear in bold are major human pathogens

As is mentioned hereinabove, an agent which specifically binds to the antitoxin binding determinant, such as a peptide or a non-biological compound can be identified using rational drug design methods, following guidance such as described, for example, by Halperin *et al.*, Proteins 47: 409–43, 2002; Gohlke and Klebe Curr Opin Struct Biol. 11: 231–235, 2001; Zeng J., Comb Chem High Throughput Screen. 3: 355–62, 2000; and RACHEL: Theory of drug design, http://www.newdrugdesign.com/Rachel_Theory.htm#Software). 3D chemical structure databases can be screened by using a suitable software such as, for example, ISIS (MDL Information Systems, San Leandro, <http://www.molinfo.com>), MACCS-3D (Martin, Y. C., J. Med. Chem. 35, 2145–2154, 1992), The Cambridge Structural Database (CSD; <http://www.ccdc.cam.ac.uk/prods/csd/csd.html>), Fine Chemical Database (reviewed in Rusinko A., 1993. Chem Des Auto. News 8, 44–47), and the NCBI's Molecular Modeling DataBase: MMDB; <http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml>.

Alternatively, an agent which specifically binds to the antitoxin binding determinant (herein refers to as the “target sequence”), such as a peptide, a polynucleotide, a carbohydrate, or a non-biological compound can be identified by high throughput screening of combinatorial libraries.

The term “library” used herein refers to a collection of chemical or biological entities which can be screened simultaneously for a property of interest.

The phrase “combinatorial library” used herein refers to a library in which the individual members are either systematic or random combinations of a limited set of basic elements, the properties of each member being dependent on the choice and location of the elements incorporated into it.

A peptide library may be prepared by either biological or non-biological synthesis methods. In a biological synthesis method, a gene encoding the peptides of interest is expressed in a host cell such that the peptides are displayed either on the surface of the cell or on the outer coat of phage produced by the cell. For example, a phage libraries can be constructed according to the protocols (Construction of Random Peptide Libraries in Bacteriophage M13 in Phage Display of Peptides and Proteins: A Laboratory Manual. Edited by B. Kay, J. Winter and J. McCafferty. Academic Press 1996.).

In order to achieve diversity, the gene must be randomized at those codons corresponding to variable residues of the peptide. It thus is not a single DNA, but rather a DNA mixture, which is introduced into the host cell culture, so that each cell has the potential, depending on which DNA it receives, of expressing any of the many possible peptide sequences of the library. The gene may be randomized by using a mixture of nucleotides rather than a pure nucleotide during appropriate synthetic cycles. The synthesis cycles may add one base at a time, or an entire codon. Examples of suitable procedures for constructing libraries of peptides generated by gene expression are described in Marks *et al.*, J Mol Biol, 222:581-597, 1991; Lam *et al.*, Nature, 354:82-84, 1991; Colas *et al.*, Nature, 380:548-550, 1996; Lu, Bio/Technology, 13:366-372, 1990; and Smith, Science, 228:1315-1317, 1985.

The peptide library may also be prepared nonbiologically by stepwise addition of amino acids. During the cycles which incorporate variable residues, the activated amino acid is chosen randomly from an amino acid mixture. Preferably, the synthesis is carried out on a solid surface, such as a pin or bead (Geyesen *et al.*, Proc Natl Acad Sci USA 81: 3998-4002, 1984), or bead (Lam *et al.*, Nature 354: 82-84, 1991).

The peptide library may be attached to a polysome using a procedure such as described, for example, in U.S. Pat. Nos. 5,643,768 and 5,658,754; Gersuk, *et al.*, Biochem. Biophys. Res. Comm. 232:578, 1997; and Mattheakis *et al.*, Proc. Nat. Acad. Sci. USA, 91:9022-9026, 1994.

If the peptide library is on a solid phase, then preferably the target sequence is tagged. Suitable tags includes, but not limited to, enzymes such as β -galactosidase, luciferase, or glutathione-S-transferase (GST) and green fluorescent protein (GFP). Other tags can be incorporated via recombinant techniques include substrate sites for enzymes such as protein kinase A which allows for the rapid and efficient labeling of the target sequence with ^{32}P . Less desirable, but still feasible, is the radio labeling of the recombinant protein, e.g., in vivo with ^{14}C or ^3H labeled amino acids or *in vitro* with ^{125}I .

If the peptide library is in solution, the target sequence may be immobilized on chromatographic media either directly [e.g., by using AFFIGEL matrix

(BioRad)], or indirectly. In indirect immobilization, the target sequence is noncovalently conjugated to the support by using an affinity reagent. For example, histidine-tagged target sequence may be immobilized on QIAGEN nickel binding resin, or a GST -tagged target sequence may be immobilized on glutathione SEPHAROSE chromatography matrix (Pharmacia). Subsequently, the immobilized target sequence is used to separate out peptides with desired activity by using methods such as described, for example, by Cantley *et al.* Trends Biochem. Sci. 20: 470-475, 1995; and Zhou and Cantley, Methods Enzymol 254: 523-535, 1995; Zhou and Cantley, Cell 72: 767-778, 1993.

In screening phage libraries, the target sequence is preferably immobilized on a solid support and screened using a procedure such as described, for example, by Devlin *et al.* (Science 249: 404-406, 1990) and Scott and Smith (Gene 128: 59-65, 1993).

Additionally, or alternatively to peptides, the agent which specifically binds to the target sequence can be a polynucleotide (aptamer). Target sequence- binding aptamers can be isolated using screening methods such as described, for example, in U.S. Pat. Nos. 5,270,163; 5,475,096; 5,567,588; 5,595,877; 5,637,459; 5,683,867; and 5,705,337 and by Colas *et al.* (Nature 380: 548-550, 1996) and Ellington and Szobtak (Nature 246: 818, 1990). For example, the starting libraries for a DNA library may be defined sequences on each end of 10 to 30 bases flanking a random core of 10 to 100 bases. Primers complementary to the defined sequences on each end are used to amplify the library and one would have a tag (such as biotin). Following amplification, the double stranded DNA is bound to a matrix (streptavidin agarose) and denatured to release ssDNA. To isolate the ligand, the target sequence is incubated with a starting library of single stranded DNA (ssDNA) and the aptamers are allowed to bind. Target-sequence and aptamer complexes are then bound onto nitrocellulose or nylon membranes and the unbound ssDNA molecules are discarded. The aptamers bound onto the target sequence are then eluted by one of several methods well known in the art (e.g., pH shock, phenol extraction, SDS treatment or heat), precipitated with ethanol and then preferably amplified by PCR to synthesize a new pool for an additional round of selection.

This process is preferably repeated 1 to 20 times. The number of repetitions

is determined by monitoring the enrichment for binders after each round or after every other round of selection. This could be accomplished in several ways. The most often used approach is to radioactively label a small percentage of the library and monitor the fraction of the library retained on the filter after each round. An alternative method is to use a primer in the amplification reaction which would allow the aptamer to be detected. Two examples of this are rhodamine and digoxigenin. Rhodamine is detected directly by fluorescence and DIG is detected by an antibody which is either directly or indirectly coupled to an enzymatic or fluorescence readout. Using a labeled primer would allow the detection of aptamer binding to target in a standard ELISA format in which the target sequence is immobilized in the well of a plate, the aptamer is added and allowed to bind and is then detected using one of the methods mentioned hereinabove. Once a sufficient level of enrichment has been attained, the final pool is amplified and cloned into a plasmid which allows for the rapid sequencing of the inserts.

Additionally, or alternatively to peptides and polynucleotides, the agent which specifically binds to the target sequence can be a carbohydrate or a small organic molecule. Libraries of carbohydrates and small organic molecules may be prepared and screened for target sequence binding activity using methods such as described, for example, by Eichler et al. (Med Res Rev. 15:481-96, 1995).

Additionally, or alternatively the agent which specifically binds to the target sequence can be a non-biological compound. Libraries of non-biological compounds may be generated and screened for target sequence binding activity using methods such as described in details in U.S. Pat. No. 6,617,114.

Agents identified capable of preventing or disrupting binding of toxin-antitoxin pairs in bacterial cells can be evaluated *in vitro* for their capacity to induce death in bacteria expressing the toxin-antitoxin pairs.

Thus, according to another aspect of the present invention, there is provided a method of identifying a molecule capable of inducing death of a bacterial cell. The method includes exposing toxin and antitoxin polypeptides of a toxin-antitoxin pair produced by the bacterial cell to a plurality of molecules followed by identifying a molecule which is capable of preventing or disrupting binding between the antitoxin and the toxin polypeptides, thereby identifying the molecule capable of inducing

death of the bacterial cell.

Preferably, exposing is effected by administering the plurality of molecules to bacteria expressing the toxin and antitoxin polypeptides, followed by determining growth of the bacteria exposed to the plurality of molecules and selecting at least one of the bacteria exhibiting a reduction in growth as compared with similar bacteria not exposed to the plurality of molecules. Preferably, the bacteria are genetically modified and cultured to overexpress the toxin-antitoxin pair, using a procedure such as described, for example, in Example 3 of the Examples section hereinbelow.

An agent identified capable of preventing or disrupting binding of toxin-antitoxin pairs in bacteria, can be used in therapy *per se* or as part (active ingredient) of a pharmaceutical composition.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal,

direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a bone tissue region of a patient.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. One route of administration which is suited for the pharmaceutical compositions of the present invention is sub-periosteal injection, as described in U.S. Pat. No. 6,525,030 to Eriksson. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose,

sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If
5 desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. As used herein, the term "oral administration" includes administration of the pharmaceutical compound to any oral surface, including the tongue, gums, palate, or other buccal surfaces. Additional methods of oral administration include
10 provision of the pharmaceutical composition in a mist, spray or suspension compatible with tissues of the oral surface.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium
15 dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a
20 plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition,
25 stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use
30 according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane,

dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (e.g. antisense oligonucleotide) effective to prevent, alleviate or ameliorate symptoms of

a disorder (e.g., mammary tumor progression) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* and cell culture assays. For example, a dose can be formulated in an animal model, such as the murine Neu model [Muller *et al.*, Cell 54, 105-115 (1988)], to achieve a desired concentration or titer.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to levels of the active ingredient which are sufficient to, for example, retard tumor progression in the case of blastic metastases (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from *in vitro* data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of

administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, 5 comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of 10 the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an 15 indicated condition, as if further detailed above.

Hence, the present invention provides methods of identifying novel antibacterial agents capable of preventing or disrupting antitoxin-toxin binding in bacterial cells, pharmaceutical compositions comprising these agents and their use in treating bacterial infections.

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Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated 25 hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the 30 above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological

and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to

which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below.

5

EXAMPLE 1

Identification of yefM and yoeB homologue genomic sequences in bacteria

Materials and methods:

Genomic sequences related to the *yefM* and *yoeB* genes of *E. coli* (SEQ ID NO: 18 and 19, respectively) were identified by a pair-constrained analysis using
 10 TBLASTN and PSI-BLAST searches (20) of non-redundant microbial genomes database at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). Putative *yefM* and *yoeB* homologue sequences were obtained and examined for constituting a toxin-antitoxin gene-pair module in the chromosome. Low homology unpaired sequences were discarded and pairs of genomic sequences positioned at distance of
 15 less than 100 bp were regarded as putative toxin-antitoxin systems.

Results:

Genomic sequence pairs related to *yefM* and *yoeB* were identified in 29 bacterial strains representing 13 different genera and 25 different species (Figure 1). It is thereby demonstrated that toxin-antitoxin systems are common and widespread
 20 among bacteria and that such systems can be identified by using bioinformatical tools such as a pair-constrained analysis of genomic sequence databases.

EXAMPLE 2

Sequence alignment of YefM and YoeB polypeptide homologues

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Materials and methods:

Multiple alignments of translated sequences of the YefM and YoeB homologues identified in Example 1 above, were produced by CLASTAL W (21) with default settings and edited using JALVIEW editor.

Results:

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Alignments of the YoeB polypeptide homologues (Figure 2B) revealed a substantially higher level of homology than was observed within the YefM and Phd family of polypeptides (Figure 2A). The relatively high degree of conservation

within the YoeB homologues is well consistent with a toxic activity that explicitly targets a specific cellular determinants and that requires a well-defined fold such as a key-lock or induced fit recognition. On the other hand, the relatively low degree of conservation of YefM and Phd homologues is consistent with a polypeptide missing a clear structural recognition and/or catalytic activity that otherwise requires a defined configuration. It should be appreciated that YefM and Phd polypeptides could be irregularly conjugated to a Doc-like or YoeB-like toxins, two families of toxins that could not be aligned and do not share any substantial homology. It is, however, consistent with a family of polypeptides which is essentially designed to be recognized as a damaged polypeptide and does not represent an interactive or catalytic scaffold.

EXAMPLE 3

Characterizing YoeB and YefM polypeptides as a toxin-antitoxin pair

Materials and methods:

Cloning YefM, YoeB and YefM-YoeB encoding sequences into pBAD-TOPO expression vector: The encoding sequences were produced by PCR using the chromosomal DNA of *E. coli* strain K-12/MC1061 as a template. The YefM encoding sequence was amplified with the primers set forth in SEQ ID NOs: 1-2. The YoeB encoding sequence was amplified with the primers set forth in SEQ ID NOs: 3-4. The YefM and YoeB pair encoding sequence was amplified using the primers of SEQ ID NOs: 1 and 4. The PCR products were inserted into the pBAD-TOPO vector to generate pBAD-yefM, pBAD-yoeB, and pBAD-yefMyoeB constructs using the pBAD-TOPO TA cloning kit (Invitrogen). The generated constructs were introduced to *E. coli* strain TOP10.

Growth rate analysis: *E. coli* TOP10 bacteria transformed with pBAD-yefM, pBAD-yoeB, and pBAD-yefMyoeB were cultured in LB broth supplemented with 100 µg/ml ampicillin (LB-Amp) and incubated at 37 °C overnight. Following incubation, cultures were diluted in fresh LB-Amp and their optical density was adjusted to approximately 0.01 (A_{600}). Each culture was then divided into two equal volumes. One volume was supplemented with 0.2% L-arabinose to induce expression of the target gene and the second volume was supplemented with 0.2%

D-glucose to suppress expression. All cultures were incubated at 37 °C and 200 rpm for up to 9 hours. Cell density was estimated periodically by optical absorbance at 600 nm. The effect of target gene induction on bacterial growth during the logarithmic growth phase was determined as described above with the exception that cultures were divided and supplemented with 0.2% L-arabinose, or 0.2% D-glucose at the time they had reached optical density of approximately 0.45 (A_{600}).

Colony formation analysis: *E. coli* TOP10 bacteria harboring pBAD-*yefM*, pBAD-*yoeB*, or pBAD-*yefMyoeB*, were cultured in LB-Amp and incubated at 37 °C overnight. Following incubation the cultures were diluted to an A_{600} of 0.01 in a fresh LB-Amp and incubated at 37 °C until an absorbance of 0.5 at A_{600} was reached. The cultures were then diluted in ten-fold dilutions steps and applied as 5 μ l droplets on LB-Amp agar plates containing L-arabinose at a concentration gradient of 0.2%, 0.1%, 0.05%, 0.02%, 0.005% and 0.0005%. An LB-Amp agar plate containing 0.2% glucose was used as a negative control. All plates were incubated at 37 °C for at least 20 hours.

Results:

Growth of bacteria overexpressing YoeB or YefM alone was substantially reduced as compared with the control (Figure 3B). On the other hand, growth of bacteria overexpressing both YefM and YoeB remained normal (Figure 3C). Similar results were observed when the expression of YoeB and YefM was induced during the logarithmic growth phase of the bacteria (Figures 3D-F). In addition, an overexpression of either YoeB or YefM alone inhibited bacterial colony formation, while, on the other hand, colony formation was unaffected when both YoeB and YefM polypeptides were overexpressed (Figure 3G).

These results clearly indicate that YoeB and YefM polypeptides behave as a toxin-antitoxin pair.

EXAMPLE 4***Biophysical characterization of YefM antitoxin******Materials and methods:***

Cloning, expression and purification of YefM from E. coli: The DNA
5 fragment containing *yefM* coding sequence flanked by primer-encoded *BsrGI* and
HindIII sites, was produced by a polymerase chain reaction (PCR) using *E. coli* K-
12 MC1061 strain chromosome as template and oligonucleotide primers set forth
in SEQ ID NOs: 5 and 2. The PCR product was digested with *BsrGI* and *HindIII*
enzymes (New England Biolabs), cloned into the *BsrGI* and *HindIII* restriction
10 sites of a pET42a expression vector (Novagen) in fusion to glutathione s-
transferase (GST) and transformed into *E. coli* BL21(DE3) pLysS (Novagen).
Transformed bacteria were cultured in 2YT broth at 37 °C and 200 rpm to an
optical density (A_{600}) of approximately 0.4. Polypeptide expression was induced
by the addition of IPTG (2 mM). One hour following induction cells were
15 harvested and re-suspended in a solution comprising phosphate buffer saline (pH
7.3), 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄), protease
inhibitor cocktail as recommended (Sigma), and 0.5 mM PMSF. The suspended
cells were lysed via three passages through a French-press cell (1400 psi) and the
insoluble material was removed by centrifugation for 20 min at 20,000 x g at 4 °C,
20 followed by filtration through a 0.45 µm membrane. The lysate supernatant was
applied onto a 1 ml glutathione sepharose column (Amersham Pharmacia Biotech)
pre-equilibrated with PBS (pH 7.3). The YefM-GST fusion protein was eluted
using 10 ml of a solution comprising 50 mM Tris-HCl (pH 8.0) and 10 mM
glutathione. YefM was separated from GST by incubation at 37 °C in the presence
25 factor Xa protease (Novagen; 16 units of protease per 1 mg YefM fusion).
Following 14 hours incubation, the protease reaction was terminated by the
addition of 1mM PMSF.

Two different methods were applied for YefM purification. In one method,
GST and linker polypeptide (~40 kDa) was separated from YefM (~11 kDa) using a
30 Sepharose HR 10/30 (FPLC) gel filtration column (Amersham Pharmacia Biotech)
and a FPLC instrument (Pharmacia LKB). Polypeptides were eluted with PBS (pH
7.3), 0.8 ml/min, and a peak that included the ~11 kDa YefM polypeptides was

collected after 13 min. Fractions containing the YefM polypeptide were completely purified using 1 μ mol of immobilized glutathione agarose (Sigma) agitated for 16 hours at room temperature. At this point, the purity of YefM was greater than 95% (estimated by Coomassie staining of SDS-PAGE).

5 In another purification method, the YefM and GST mixture was divided into 0.5 ml aliquots, boiled for 10 minutes and then centrifuged at 14,000 rpm for 10 minutes. The supernatants, containing purified YefM, were collected and united.

The YefM concentration was estimated based on tyrosine absorbance in 0.1M KOH was used. Polypeptide concentrations were calculated using the
10 extinction coefficients of 2391 $M^{-1} cm^{-1}$ (293.2 nm in 0.1 M KOH) for single tyrosine.

The molecular mass of YefM was verified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a voyager-DE STR Biospectrometry workstation (Applied Biosystems) and using α -Cyano-4-
15 hydroxycinnamic acid as the matrix.

Circular Dichroism (CD): CD spectra were obtained by using an AVIV 202 spectropolarimeter equipped with temperature-controlled sample holder and a 5 mm path length cuvette. Mean residual ellipticity, $[\theta]$, was calculated as,

$$[\theta] = [100 \times \theta \times m] / [c \times L]$$

20 where θ is the observed ellipticity, m is the mean residual weight, c is the concentration in mg/ml, and L is the path length in centimeters.

All experiments were performed in PBS (pH 7.3) at polypeptide concentration of 10 μ M. For thermal denaturation experiments, samples were equilibrated at each temperature for 0.5 min and CD ellipticity at 222 nm and 217
25 nm was averaged for 1 min.

Fourier Transform Infrared Spectroscopy (FTIR): Spectra were recorded using a Nicolet Nexus 470 FT-IR spectrometer with a DTGS detector. A sample of 1 μ g of lyophilized YefM, suspended in 30 μ l PBS in D₂O (pH 7.3), was dispensed on a CaF₂ plate. Spectrometer measurements were taken using a 4 cm^{-1} resolution
30 and 2,000 scans averaging and the transmittance minima values were determined by the OMNIC analysis program (Nicolet).

Amino acid composition and charge-hydrophobicity values analysis: The occurrence rate of each amino acid in the YefM family of polypeptides (P_{Mi}) was determined by averaging its 30 frequencies in 30 YefM homologue sequences. The general amino acid occurrence rates (P_{Gi}) were compiled by the Rockerfeller authors using the NCBI database (22). The relative differences between the occurrence rates of amino acids in YefM polypeptides and in general polypeptides was evaluated as $(P_{Mi} - P_{Gi}) / P_{Gi}$. The variances of these ratios were calculated as: $\text{Var}(P_{Mi}) / (P_{Gi})^2$. The overall hydrophobicity and net charge values of YefM family of polypeptides were calculated and plotted according to Uversky *et al.* (3).

YefM stability analysis: Overnight culture of *E. coli* carrying the pBAD-yefM plasmid was grown at 37 °C /200 rpm in LB broth to stationary phase ($\text{OD}_{600} = 1.4$). YefM expression was then induced for 10 min with 0.2% L-arabinose and subsequently treated with 200 µg/ml rifampicin and 0.2% glucose to repress further expression from pBAD promoter. Aliquots of 2 ml were removed before and at 15 min intervals after repression, and the cellular YefM polypeptide was quantitatively analyzed by western blot. Densitometer assessment of YefM was effected using ImageScanner (Amersham Biosciences) and the ImageMaster 1D prime (ver. 3.01) program (Amersham Biosciences).

Results:

A far UV circular dichroism (CD) spectra of the purified YefM polypeptide (in both purification methods) at increasing temperatures (25, 37 and 42 °C) is illustrated in Figure 4A. The spectra indicate a typical random-coil pattern, according to Jenness *et al.* (25), with a minimum in the vicinity of 200 nm with only slight changes in spectra due to temperature increase. An FTIR spectrum of the purified YefM at room temperature is illustrated in Figure 4B. The spectrum shows a transmittance minimum at 1643 cm^{-1} which is indicative of a random-coil structure according to Haris and Chapman (26). Thermal denaturation curve further indicates a consistent predominant random-coil structure of YefM which is maintained at 2 °C to 80 °C range (Figure 4C). The unfolded state of YefM is further supported by its extraordinary solubility during boiling (Figure 4D).

In order to get insight into the structural stability of the YefM in its native state within cells, a short expression of YefM was effected followed by its full

repression under stationary growth. Analysis of YefM levels in *E. coli*, before and after repression at different intervals, revealed that native YefM has an *in vivo* half-life of approximately one hour (Figure 7), which is characteristic to an antitoxin.

These results clearly indicate that YefM is an unfolded and unstable polypeptide *in vivo*.

EXAMPLE 5

Biophysical characterization of YoeB toxin

Materials and methods:

Cloning, expression and purification of YoeB from *E. coli*: The DNA fragment containing *yefM-yoeB* coding sequence was produced by a polymerase chain reaction (PCR) using *E. coli* K-12 MC1061 strain chromosome as template and oligonucleotide primers set forth in SEQ ID NOs: 5 and 132. The PCR product was cloned into the pTrcHis2 expression vector (Invitrogen) in fusion to myc-epitope and his-tag, to generate pTMB. The plasmid was transformed into *E. coli* TOP10 strain (Invitrogen). Transformed bacteria were cultured in 2YT broth at 37 °C and 200 rpm to an optical density (A_{600}) of approximately 0.4. Polypeptide expression was induced by the addition of IPTG (2 mM). Following one hour induction, cells were harvested and re-suspended in a Buffer A (phosphate buffer saline, pH 8.0; 50 mM $\text{Na}_2\text{HPO}_4\text{-NaOH}$; 0.3 M NaCl; and 0.5 mM PMSF). The suspended cells were lysed via three passages through a French-press cell (1400 psi) and the insoluble material was removed by centrifugation for 20 min at 20,000 x g at 4 °C, followed by filtration through a 0.45 μm membrane. The lysate supernatant was applied onto a Ni-CAM HC resin (Sigma) packed in a XG 16/20 FPLC column (Amersham Biosciences) pre-equilibrated with Buffer A. Following column wash, polypeptides were eluted with buffer A and 250 mM imidazole solution, in a single broad peak representing the purified YoeB-His (in a small number of fractions) or YoeB-His together with YefM (in most fractions). When eluted alone, YoeB-His purity was at least 90% as estimated by SDS-PAGE Coomassie-blue staining.

Eluted YefM and YoeB-His complex was applied to HiPrep 16/10 Q XL column and pre-equilibrated with Buffer B (20 mM Tris-HCl, pH 8.0). Elution was

performed with developing NaCl gradient from 0.02 to 1 M in buffer B. The YefM : YoeB-His complex was eluted at approximately 650 mM NaCl. The complex was also separated using an analytic reverse-phase C₁₈ column (Vydac) with increasing gradient of 0 to 80% acetonitrile and 0.1% trifluoroacetic acid. YefM and YoeB-His polypeptides were eluted in a single peak in approximate 50% acetonitrile. In all above cases, elution was monitored at A₂₈₀.

The identity of YefM and YoeB-His was verified using protein spots isolation from Coomassie blue stained gels and was accomplished by mass spectrometry, according to established protocols (Bandow, J.E., Becher, D., Buttner, K., Hochgrafe, F., Freiberg, C., Brotz, H. and Hecker, M. (2003) *Proteomics*, **3**, 299-306). Briefly, protein spots were excised from stained gels and the gel pieces were treated trypsin solution (Pomega) for 16 h at 37 °C. Peptides were extracted from gel onto a sample plate for MALDI-MS. Obtained peptides masses were determined in the positive ion reflector mode in a Voyager-DE STR mass spectrometer (Applied Biosystems). Peptide mass fingerprints were compared to databases using the MS-Fit program (<http://prospector.ucsf.edu>). To determine the concentrations of YoeB-His and YefM polypeptides, tyrosine and tryptophan absorbance measurements in 0.1 M KOH were used. YefM concentration was calculated using the extinction coefficient of 2381 M⁻¹cm⁻¹ at 293.2 nm for single tyrosine (4 tyr). YoeB-His concentration was calculated using extinction coefficient at 280 nm of 1507 M⁻¹cm⁻¹ for single tyrosine (6 tyr) and 5377 M⁻¹cm⁻¹ for single tryptophan (4 trp). Circular Dichroism (CD) spectra were obtained as described in Example 4 hereinabove. For thermal denaturation experiments, the sample temperature was equilibrated for 30 sec at each temperature interval and the ellipticity at 222 nm was averaged for 1 min. All experiments were performed in PBS, pH 7.3.

Results:

The YoeB-His and YefM polypeptides co-eluted using nickel column chromatography (Figure 9A), ion-exchange chromatography on a Q-sepharose column (Figure 9B) and RP-HPLC chromatography using a C₁₈ analytical column (Figure 9C), indicating a toxin-antitoxin complex.

The structure of purified YoeB-His toxin, as indicated from the far-UV

circular dichroism (CD) spectrum (Figure 10A), is consistent with a well-folded protein containing at least 50% α -helical secondary structure. The secondary structure content of the toxin remains nearly unchanged between 4 - 37 °C. A near-UV CD analysis of YoeB-His also showed stability over this temperature range (Figure 10B) indicating that the tertiary structure of the toxin remains virtually unchanged as well. In addition, the YoeB-His exhibits full structural reversibility within the 4 – 37 °C temperature range (Figure 10A).

In order to determine the thermodynamic stability, YoeB-His underwent thermal denaturation between 2 and 80 °C monitored by CD ellipticity at 222 nm in neutral buffer. As can be seen in Figure 10C, a sharp increase in ellipticity slope was observed during the melting phase at approximately 60 °C. Cooling back from 80 °C to 2 °C could not bring to any observed renaturation, indicating that YoeB-His polypeptide could not regain its native conformation following such melt. In order to identify the melting point of the toxin, YoeB-His was partially melted and then cooled back repeatedly, each time raising the target temperature in about 5 °C increments. Conformational changes were monitored by measuring the CD ellipticity at 222 nm. The analysis shows structural reversibility of YoeB-His at a temperature ranging from 4 to 56 °C (Figures 11A-B). However, the YoeB-His polypeptide was unable to refold following melting at 60 °C (Figure 11C), indicating that the polypeptide melting point (T_M) is approximately 60 °C.

EXAMPLE 6

Identification of YefM recognition determinants

Materials and methods:

Cloning, expression, and purification of YefM from *E. coli*: performed as described in Example 4 above.

Cloning, expression, and purification of GST-YoeB from *E. coli*: DNA fragment containing the coding sequence of *yoeB*, flanked by primer-encoded *EcoRI* and *HindIII* sites, was produced by a polymerase chain reaction using *E. coli* K-12 MC1061 strain chromosome as template and oligonucleotide primers set forth in SEQ ID NOs:1 and 132. The PCR product was digested with *EcoRI* and *HindIII* enzymes (New England Biolabs), cloned into the *EcoRI* and *HindIII* restriction sites

of the pET42a expression vector in fusion to GST, and transformed into *E. coli* BL21(DE3) pLysS. Bacteria were grown, expressed and lysed in the same manner described above for GST-YefM fusion protein. The supernatant was applied onto a 1 ml glutathione sepharose column (Amersham Pharmacia Biotech) pre-equilibrated with PBS (pH 7.3). The bound protein was eluted using 10 ml of 50 mM Tris-HCl (pH 8.0), 10 mM glutathione. Eluted fractions containing the GST-YoeB fusion protein were collected and quantitatively assessed by Coomassie staining of SDS-PAGE.

Peptide array analysis: Tridecamer peptides corresponding to consecutive overlapping sequences of the YefM polypeptide were arrayed on a cellulose membrane matrix and covalently bound to a Whatman 50 cellulose support (Whatman). Approximately 50 µg aliquots of soluble GST-YoeB fusion were examined for their selective peptide binding ability, on the basis of YefM-YoeB putative interaction.

For low stringency binding the cellulose membrane was briefly washed in 100% ethanol, washed three times with Tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.5, 150 mM NaCl), then blocked for 4 hours using 5% (w/v) non-fat milk in TBS. The membrane was then washed three times in TBS + 0.1% (v/v) tween 20 (TBS-T) and incubated with 10 ml GST-YoeB solution at 4 °C and slow shaking for 14 hours.

For high stringency binding the washing steps were extensive and multiple and the blocking solution washing step was reduced to a single brief wash.

Following incubation, the membrane was washed once in TBS-T then supplemented with 10 ml suspension comprising TBS, mouse anti-GST antibody and horseradish peroxidase conjugated goat anti-mouse antibody in the appropriate titers. Following 1 hour incubation at room temperature the membrane was briefly washed with TBS-T and TBS. Bound GST-YoeB proteins were detected through an enhanced chemiluminescence reaction following an exposure to a sensitive film.

Western blot analysis: Bacterial culture aliquots (2 ml) were centrifuged at 14,000 rpm for 5 min at 4°C and re-suspended in 80 µl of double-distilled water. Sixty µl suspension aliquots were added to 20 µl aliquots of 4 x sample buffer, and the remaining 20 µl aliquots were used to quantify the total polypeptide using the

Coomassie plus protein assay reagent (Pierce). Aliquots containing equal total polypeptide amounts were loaded on a tris-tricine SDS 15% polyacrylamide slab gel. After electrophoresis, the proteins were electroblotted to PVDF membrane filters (Bio-Rad) and exposed to anti-YefM serum raised in rabbit. The membrane was then incubated with peroxidase-conjugated anti-rabbit antibodies and the presence of YefM was determined by an enhanced chemiluminescence reaction following by an exposure to a sensitive film.

Surface plasmon resonance analysis: Binding affinities were evaluated by surface plasmon resonance (SPR) using BIAcoreTM2000 (BIAcore Inc., NJ). Approximately 30 resonance units of the peptide having the amino acid sequence set forth in SEQ ID NO: 7, denoting to the YefM antitoxin binding determinant sequence, was immobilized onto a research grade sensor chip CM5 using amine coupling kit (BIAcore) as described by the manufacturer. Suspensions of 12.5, 25, and 50 nM GST-YoeB fusion polypeptide in 50 mM Tris (pH 7.2) were passed over the chip surface at room temperature and a flow rate of 10 μ l / min. The chip surface was regenerated with 10 mM HCl after each run and re-equilibrated with Tris buffer. Sensogram data were analyzed using the BIAevaluation 3.0 software package. The rate constants were calculated for the binding data using local fitting for the data set as described in the BIAevaluation 3.0 manual with the 1:1 Langmuir binding model.

Results:

Three putative YefM fragments capable of binding GST-YoeB fusion protein were identified in a peptide array using a low stringency procedure (Figure 6A). A first region comprises three tridecamer peptides (YefM₁₁₋₂₃–YefM₁₅₋₂₇) in decreasing binding capacity, which includes the sequence RTISYSEARQNLSATMM (the underlined sequence represents a putative binding site; set forth in SEQ ID NO: 129). A second region includes a single YefM₃₃₋₄₅ peptide sequence, APILITRQNGEAC (set forth in SEQ ID NO: 130). A third region includes the peptides YefM₇₅₋₈₇ and YefM₇₇₋₈₉ which cover the MDSIDSLKSGKGTEKD (set forth in SEQ ID NO: 131). In a high stringency procedure the examined sites were extended to include YefM₈₋₃₁ as the first region, YefM₂₉₋₄₈ as the second region and YefM₇₂₋₉₂ as the third region. The shift between each arrayed tridecamer peptide was reduced to a single amino acid which resulted in identifying the YefM₁₁₋₂₃

peptide, having the amino acid sequence set forth in SEQ ID NO: 7, as a sequence containing the antitoxin binding determinant (Figure 6B).

In replacing the amino acid leucine in position 22 of the YefM₁₁₋₂₃ peptide to alanine or to glycine only attenuated the binding capacity of GST-YoeB. On the other hand, replacing the amino acid arginine in position 19 of the YefM₁₁₋₂₃ peptide, with either alanine or glycine (set forth SEQ ID NOs: 8 and 9, respectively) totally negated the binding capacity of the YefM₁₁₋₂₃ peptide analog with GST-YoeB (Figure 6C).

Surface plasmon resonance (BIAcore) analysis was used to quantitatively determine the affinity between the YoeB toxin and the YefM₁₁₋₂₃ peptide fragment. The recognition determinant sequence peptides were immobilized onto the sensor chip and the kinetics of GST-YoeB binding and dissociation was estimated at 12.5, 25, and 50 nM concentrations (Figure 8). According to data analysis, a k_a of 3.06×10^3 ($M^{-1}s^{-1}$) and a k_d of 1.22×10^3 ($M^{-1}s^{-1}$) were calculated (arithmetic mean). Accordingly, an equilibrium constant (K_D) of 0.4 μM was determined for the YoeB-YefM₁₁₋₂₃ complex. This dissociation constant is consistent with a specific binding between the toxin and the peptide fragment.

The isolated binding determinant of the YefM antitoxin can be utilized to identify agents capable of preventing or disrupting the YoeB-YefM toxin-antitoxin binding and thereby to induce death of bacteria expressing the YoeB and YefM toxin-antitoxin pair.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit

and broad scope of the appended claims. All publications, patents, patent applications and sequences identified by their accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent, patent application or sequence identified by their accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

REFERENCES CITED

(Additional references are cited hereinabove)

1. Anfinsen, C.B. (1973) *Science* **181**, 223-230.
2. Schweers, O., Schönbrunn-Hanebeck, E., Marx, A. and Mandelkow, E. (1994) *J. Biol. Chem.* **269**, 24290-24297.
3. Uversky, V.N., Gillespie, J.R. and Fink, A.L. (2000) *Proteins Struct. Funct. Genet.* **41**, 415-427.
4. Uversky, V.N. (2002) *Prot. Sci.* **11**, 739-756.
5. Uversky, V.N. (2002) *Eur. J. Biochem.* **269**, 2-12.
6. Dunker, A.K., Lawson, J.D., Brown, C.J. Williams, R.M., Romero, P., Oh, J.S., Oldfield, C.J., Campen, A.M., Ratliff, C.M., Hipps, K.W., Ausio, J., Nissen, M.S., Reeves, R., Kang, C.-H., Kissinger, C.R., Bailey, R.W., Griswold, M.D., Chiu, W., Garner, E.C. and Obradović, Z. (2001) *J. Mol. Graph. Model.* **19**, 26-59.
7. Dobson, C.M. (1999) *Trends Biochem. Sci.* **24**, 329-332.
8. Rochet, J.C. and Lansbury, P.T.Jr. (2000) *Curr. Opin. Struct. Biol.* **10**, 60-68.
9. Gazit, E. (2002) *Curr. Med. Chem.* **9**, 1725-1735.
10. Dunker, A.K., Brown, C.J., Lawson, J.D., Iakoucheva, L.M. and Obradović, Z. (2002) *Biochemistry* **41**, 6573-6582.
11. Gazit, E. and Sauer, R.T. (1999) *J. Biol. Chem.* **274**, 2652-2657.
12. Lehnherr, H., Maguin, E., Jafri, S. and Yarmolinsky, M.B. (1993) *J. Mol. Biol.* **233**, 414-428.
13. Lehnherr, H. and Yarmolinsky, M.B. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 3274-3277.
14. Christensen, S.K. and Gerdes, K. (2003) *Mol. Microbiol.* **48**, 1389-1400.
15. Pedersen, K., Zavialov, A.V., Pavlov, M.Y., Elf, J., Gerdes, K. and Ehrenberg, M. (2003) *Cell* **112**, 131-140.
16. Pedersen, K., Christensen, S.K. and Gerdes, K. (2002) *Mol. Microbiol.* **45**, 501-510.
17. Christensen, S.K., Mikkelsen, M., Pedersen, K. and Gerdes, K. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 14328-14333.

18. Gerdes, K. (2000) *J. Bacteriol.* **182**, 561-572.
19. Aizenman, E., Engelberg-Kulka, H. and Glaser, G. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 6059-6063.
20. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) *Nucleic Acids Res.* **25**, 3389-3402.
21. Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) *Nucleic Acids Res* **22**, 4673-4680.
22. <http://prowl.rockefeller.edu/aainfo/masses.htm>.
23. Pomerantsev, A.P., Golovliov, I.R., Ohara, Y., Mokrievich, A.N., Obuchi, M., Norqvist, A., Kuoppa, K. and Pavlov, V.M. (2001) *Plasmid* **46**, 210-222.
24. Grady, R. and Hayes, F. (2003) *Mol. Microbiol.* **47**, 1419-1432.
25. Jeness, D.D., Sprecher, C.A. and Johnson, W.C. (1976) *Biopolymers* **15**, 513-521.
26. Gazit, E. and Sauer, R.T. (1999) *J. Biol. Chem.* **274**, 16813-16818.
27. Oberer, M., Lindner, H., Glatter, O., Kratky, C. and Keller, W. (1999) *Biol. Chem.* **380**, 1413-1420.
28. Thi, M.H.D., Messens, J., Wyns, L. and Backmann, J. (2000) *J. Mol. Biol.* **299**, 1373-1386.
29. Camacho, A.G., Misselwitz, R., Behlka, J., Ayora, S., Welfle, K., Meinhart, A., Lara, B., Saenger, W., Welfle, H. and Alonso, J.C. (2002) *Biol. Chem.* **383**, 1701-1713.
30. Romero, P., Obradović, Z., Li, X., Garner, E.C., Brown, C.J. and Dunker, K. (2001) *Proteins Struct. Funct. Genet.* **42**, 38-48.
31. Vihinen, M., Torkkila, E. and Riikonen, P. (1994) *Proteins* **19**, 141-149.
32. Radivojac, P., Obradović, Z., Brown, C.J. and Dunker, A.K. (2003) *Pac. Symp. Biocomput.* **8**, 216-227.